

Original articles

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15-DEOXY- $\Delta^{12,14}$ -PROSTAGLANDIN J_2 SUPPRESSES RANTES EXPRESSION BY INHIBITING NADPH OXIDASE ACTIVATION IN *HELICOBACTER PYLORI*-INFECTED GASTRIC EPITHELIAL CELLS

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Peroxisome proliferators-activated receptor- γ (PPAR- γ) is a ligand-activated transcription factor. 15 deoxy- $\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ₂) is a potent PPAR- γ ligand and acts as an anti-inflammatory agent *via* PPAR- γ -dependent and independent mechanisms. *Helicobacter pylori* (*H. pylori*) induces gastric inflammation by inducing the activation of oxidant-sensitive transcription factor NF- κ B and cytokine expression in gastric epithelial cells. Since 15d-PGJ₂ inhibits NF- κ B activation in various cells, it may suppress *H. pylori*-induced inflammatory signaling and cytokine expression in gastric epithelial cells. The present study aims to determine the effect of 15d-PGJ₂ on the activation of inflammatory mediators Jak/Stat (Janus kinase/signal transducers and activators of transcription) and induction of cytokine RANTES in *H. pylori*-infected gastric epithelial AGS cells. Since NADPH oxidase is a candidate for the production of reactive oxygen species in *H. pylori*-infected gastric epithelial cells, we determined the effect of 15d-PGJ₂ on the activation of NADPH oxidase. AGS cells were cultured in the presence of *H. pylori* treated with or without 15d-PGJ₂. The activations of NADPH oxidase and Jak1/Stat3, the levels of H₂O₂ and RANTES in the medium, and DNA binding activity of Stat3 were assessed. A Jak/Stat3 specific inhibitor AG490 and an inhibitor of NADPH oxidase diphenyleneiodonium (DPI) were treated to determine the direct involvement of Jak/Stat and NADPH oxidase on the production of H₂O₂ and RANTES in *H. pylori*-infected cells. *H. pylori* induced the production of H₂O₂ and RANTES as well as the activations of NADPH oxidase and Jak1/Stat3, which were inhibited by the treatment of 15d-PGJ₂. DPI suppressed *H. pylori*-induced alterations similar to 15d-PGJ₂. However, AG490 had no effect on NADPH oxidase activation, but reduced the level of RANTES in the medium released from *H. pylori*-infected cells. Conclusion: NADPH oxidase activation is an upstream signaling of Jak1/Stat3 activation and induction of RANTES in *H. pylori*-infected AGS cells. 15d-PGJ₂ inhibits the activations of NADPH oxidase and Jak1/Stat3 and RANTES expression, suggesting that 15d-PGJ₂ may be beneficial for the treatment of *H. pylori*-induced gastric inflammation.

Key words: 15 deoxy- $\Delta^{12,14}$ prostaglandin J_2 , human stomach adenocarcinoma (AGS) cells, *Helicobacter pylori*, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, RANTES

INTRODUCTION

The microaerophilic bacterium, *Helicobacter pylori* (*H. pylori*), plays an important role in the pathogenesis of chronic gastritis, peptic ulcer, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (1-5). The epithelial cytokine/chemokine response is particularly important in the early stages of *H. pylori*-induced inflammation. RANTES (short for "regulated upon activation, normal T cell expressed and secreted") is a potent chemotactic agent for T lymphocytes and monocytes (6) and expressed in fibroblasts, T cells, monocytes, endothelial cells, and epithelial cells after inflammatory stimuli. Therefore, RANTES may contribute to the infiltration of inflammatory cells into gastric tissues infected with *H. pylori*. Indeed, *H. pylori* induced the expression of RANTES in gastric epithelial cells (7), which may activate infiltrating inflammatory cells such as lymphocytes into the infected tissues. Reactive oxygen species (ROS) are produced in

the activated inflammatory cells of the infected tissues (8) and gastric epithelial cells infected with *H. pylori* (9-11).

One of the main sources of ROS in phagocytic and non-phagocytic cells is NADPH oxidase. Activation of NADPH oxidase requires the assembly of membrane-integrated cytochrome b558 (a heterodimer formed by gp91^{phox} and p22^{phox}) with cytosolic components p47^{phox}, p67^{phox}, and the small GTPase Rac (12). During the activation of NADPH oxidase, Rac translocates to the membrane and GTP-bound Rac directly interacts with p67^{phox} and p47^{phox} (13, 14). We recently found that *H. pylori* induces translocation of HSP90 β from cytosol to membrane (15). In the membrane, HSP90 β interacts with Rac1, which activates NADPH oxidase to produce ROS in *H. pylori*-infected gastric epithelial cells. Therefore, NADPH oxidase may be an upstream signaling mediator in *H. pylori*-induced gastric inflammation.

Jak/Stat (Janus kinase/signal transducers and activators of transcription) signaling cascade functions as one of the major

immune and cytokine signals (16, 17). The binding of inflammatory ligand such as cytokine to its receptor induces the assembly of an active receptor associated Jaks (Jak1, Jak2, Jak3, and TYK2). Phosphorylated Jaks lead to the activation of neighboring Jaks, receptor subunits, and several other substrates. Phosphorylation of Jak provides the docking sites for Stats, which in turn become phosphorylated on tyrosine and serine residues; the phosphorylations of both amino acid species are required for full Stat activity. Phosphorylated Stats are released from the receptor complex and form dimers. These dimers translocate to the nucleus where they directly bind to the promoter region of specific target genes, thus regulating transcription of these genes involved in immune responses (18-20). Therefore, it is possible that *H. pylori*-stimulated inflammatory signaling includes Jak/Stat activation. However, the role of Jak/Stat on *H. pylori* infection has not been investigated yet.

15deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) is a natural ligand that activates the peroxisome proliferators-activated receptor- γ (PPAR- γ), a member of nuclear receptor family. 15d-PGJ₂ stimulates transcription of target genes via PPAR- γ -dependent mechanism (21). It exerts anti-inflammatory activities in macrophages and endothelial cells via PPAR- γ -dependent and independent mechanisms (22-25). The recent studies demonstrated that 15d-PGJ₂ has anti-inflammatory action by inhibiting the activation of NF- κ B (26-28) and mitogen-activated protein kinases (MAPK) (28), and the induction of cytokines and chemokines (28, 29). 15d-PGJ₂ inhibited IFN γ -induced Jak/Stat signaling pathway independent of PPAR- γ (30). 15d-PGJ₂ and other PPAR- γ ligand ciglitazone inhibited O₂⁻ production by suppressing the expression of NADPH oxidase, a major source of O₂⁻ in vascular endothelial cells (31). Previously, we found that *H. pylori* stimulates the production of ROS (9-11) and induces the expression of cytokines by activating NF- κ B and MAPK in gastric epithelial cells (32-34). Therefore, 15d-PGJ₂ may inhibit NADPH oxidase activation and thus, suppress ROS production and inflammatory signaling in *H. pylori*-infected gastric epithelial cells. Recently it has been reported that *H. pylori* induced nuclear translocation of PPAR- γ in gastric epithelial cells (35).

In the present study, we determined whether 15d-PGJ₂ inhibits the activation of NADPH oxidase and inflammatory mediators Jak/Stat and induction of RANTES in *H. pylori*-infected gastric epithelial AGS cells. A Jak/Stat3 specific inhibitor AG490 and an inhibitor of NADPH oxidase diphenyleneiodonium (DPI) were treated to the cells for investigating the direct involvement of Jak/Stat and NADPH oxidase on the production of H₂O₂ and RANTES in *H. pylori*-infected cells.

MATERIALS AND METHODS

Reagents

Reagents for nitrocellulose membranes were obtained from Amersham Inc. (Arlington Heights IL, USA). Anti-phosphotyrosine-Stat3 (Tyr⁷⁰⁵) antibody was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Polyclonal anti-Stat3 antibody was purchased from Upstate (Charlottesville, VA, USA). Antibodies against phospho-Jak1, Jak1, p47^{phox}, p67^{phox}, actin and anti-rabbit and anti-goat secondary antibodies were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂, PPAR- γ ligand), Tyrphostin AG490 (Jak/Stat3 specific inhibitor), and diphenyleneiodonium (DPI, NADPH oxidase inhibitor) were purchased from Calbiochem (La Jolla, CA, USA). 15d-PGJ₂,

AG490 and DPI were dissolved in dimethylsulfoxide (DMSO) and pretreated to AGS cells at final concentrations of 10 μ M (15d-PGJ₂), 40 μ M (AG490), and 10 μ M (DPI) 2 hours prior to *H. pylori* treatment. Xylenol orange and β -nicotinamide adenine dinucleotide phosphate reduced form (β -NADPH) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Human gastric epithelial AGS cells (gastric adenocarcinoma, ATCC CRL 1739) were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown until 70-80% confluent in RPMI-1640 medium (pH 7.4; Sigma-Aldrich) supplemented with 10% fetal bovine serum, 4 mM glutamine (GIBCO-BRL, Grand Island, NY, USA) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin). The cells were used for the experiments 20 hours after seeding at 37°C in a humidified atmosphere of 95% air-5% CO₂.

Helicobacter pylori culture and infection

An *H. pylori* strain used in the present study is HP99 which was isolated from Korean patients and identified as cagA+, vacA+ strain (34). HP99 is kindly provided from Dr. H.C. Jung (Seoul National University College of Medicine, Seoul, Korea). These bacteria were inoculated onto chocolate agar plates (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) at 37°C under microaerophilic conditions using an anaerobic chamber (BBL Campy Pouch® System, Becton Dickinson Microbiology Systems). *H. pylori* suspended in antibiotic-free cell culture medium were added to AGS cells at a bacterium/cell ratio of 300:1, based on the previous studies (9, 11, 36).

Determination of H₂O₂ in the medium

The cells (3x10⁵/ml) were cultured in the presence or absence of *H. pylori* for 15, 30, and 60 min. Levels of H₂O₂ in the medium were determined by xylenol orange (XO) assay (37). At each time point, the medium was collected and centrifuged to eliminate bacteria and cell debris. 20 μ l of supernatant was added to 200 μ l of XO mixture (containing 125 μ M XO, 100 mM D-sorbitol, 250 μ M FeSO₄, 250 μ M (NH₄)₂SO₄, 25 mM H₂SO₄). Sample/XO mixture was reacted for 30 min at room temperature. Absorbance at 560 nm (A₅₆₀) of sample/XO mixture was measured against a blank prepared with 20 μ l fresh medium containing 200 μ l of XO mixture. A standard curve was prepared with different concentrations of H₂O₂. For investigating the effects of 15d-PGJ₂, AG490, and DPI on *H. pylori*-induced increase in H₂O₂ production, the cells were treated with 15d-PGJ₂, AG490, and DPI for 2 hours, and cultured in the presence of *H. pylori* for 30 min.

Preparation of extracts

The cells (3.5x10⁶/ml) were cultured in the presence or absence of *H. pylori* for 15, 30, 60 (for activity and activation of NADPH oxidase), or 120 min (for Jak/Stat activation). The cells were rinsed with ice-cold PBS, harvested by scraping into PBS and pelleted by centrifugation at 1,500 rpm for 5 min. The cells were suspended with lysis buffer containing 10 mM Tris, pH 7.4, 15 mM NaCl, 1% NP-40, complete (protease inhibitor complex, Roche, Mannheim, Germany), extracted by drawing the cells through a 1-ml syringe with several rapid strokes, left on ice for 30 min, and centrifuged at 13,000 rpm for 10 min. The supernatant was collected and used as a whole cell extract. To prepare the cytosolic and membrane fractions, the supernatant was separated further by centrifugation at

100,000 g for 1 hour. The membrane fraction was obtained by suspending the pellet with lysis buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol. The supernatant was used as the cytosolic fraction. The protein concentration was determined by the Bradford method (38) using Bio-Rad protein assay solution (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For determining the effects of 15d-PGJ₂, AG490, and DPI on *H. pylori*-induced increase in NADPH oxidase activity, activation of NADPH oxidase, and Jak/Stat activation, the cells were treated with 15d-PGJ₂, AG490, and DPI for 2 hours and cultured in the presence of *H. pylori* for 15 min (for activity and activation of NADPH oxidase) or 30 min (for Jak/Stat activation).

Measurement of NADPH oxidase activity

NADPH oxidase activity was measured by lucigenin assay. The membrane and cytosolic fractions were prepared as described previously. The assay was performed in 50 mM Tris-Mes buffer, pH 7.0, containing 2 mM KCN, 10 μ M lucigenin and 100 μ M NADPH as the substrate. The reaction was started by addition of membrane fractions containing 10 μ g proteins. The photon emission was measured every 15 s for 5 min in a microtiterplate luminometer (MicroLumat LB 96V luminometer, Berthold, NH, USA). NADPH oxidase activity was also monitored by addition of cytosolic fractions to the reaction mixture as a negative control. As a reference experiment, to determine the possible artifacts by lucigenin concentration, the membrane fractions were prepared from the cells cultured in the absence of *H. pylori*. ROS production was monitored by addition of membrane fraction to a reaction mixture containing NADPH and the concentration of 10 μ M lucigenin. The photon emission was measured every 15 s for 5 min in a microtiterplate luminometer.

Western blot analysis

The proteins of whole cell extract, cytosolic fraction, and membrane fraction were loaded, separated by 8-12% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes by electroblotting. After blocking of nonspecific binding with 5% nonfat dry milk in TBS-T (Tris-buffered saline and 0.15% Tween 20) for 2 hours at room temperature, the membranes were probed with primary antibody (phospho-Jak1, Jak1, phospho-Stat3, Stat3, p47^{phox}, and p67^{phox}) at 4°C overnight. Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (anti-goat or anti-rabbit), respectively, and visualized by the ECL detection system (Santa Cruz Biotechnology) according to the manufacturer's instruction. Actin served as a loading control.

Nuclear extracts preparation and electrophoretic mobility shift assay (EMSA) for Stat3-DNA binding activity

The cells (3.5x10⁶/ml) were treated with or without 15d-PGJ₂, AG490, and DPI for 2 hours and cultured in the presence of *H. pylori* for 30 min. Nuclear extracts were prepared as described previously (33). Stat3 gel shift oligonucleotide (5'-GATCCTTCTGGAATTCCTAGATC-3') from Santa Cruz Biotechnology were labeled with [³²P] dATP (Amersham Inc.) using T4 polynucleotide kinase (GIBCO-BRL). End-labeled probe was purified from unincorporated [³²P] dATP using a purification column (Bio-Rad Laboratories) and recovered in Tris-EDTA buffer (TE). Nuclear extracts (3 μ g) were pre-incubated in buffer containing 12% glycerol, 12 mM HEPES, pH 7.9, 4 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM DTT, 25 mM KCl, 5 mM MgCl₂, 0.04 μ g/ml poly [dI-dC] (Amersham Inc.), 0.4 mM PMSF, and TE. The labeled probe was added and

samples were reacted on room temperature for 30 min. Samples were loaded, separated by electrophoresis on a non-denaturing 5% polyacrylamide gel at 200 mM using 0.5X TBE buffer. The gels were dried at 80°C for 30 min and exposed to the radiography film for 6-18 hours at -70°C with intensifying screens (39).

Determination of RANTES in the medium

The cells (5x10⁵/ml) were cultured in the presence or absence of *H. pylori* for 4, 8, 12, and 24 hours. The levels of RANTES in the medium were determined by enzyme-linked immunosorbent assay (ELISA, Biosource, Camarillo, CA, USA). For determining the effects of 15d-PGJ₂, AG490, and DPI on *H. pylori*-induced production of RANTES, the cells were treated with 15d-PGJ₂, AG490, and DPI for 2 hours and cultured in the presence of *H. pylori* for 4 hours.

Statistical analysis

The statistical differences were determined using one-way ANOVA by Newman-Keul's test. All values are expressed as a mean S.E. of four different experiments. A value of $p < 0.05$ was considered statistically significant.

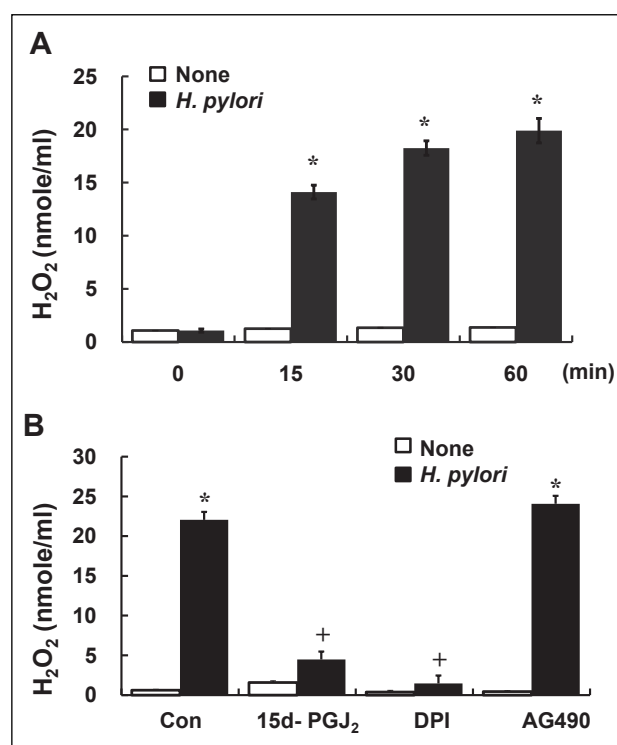


Fig. 1. H₂O₂ levels in the medium released from *H. pylori*-infected AGS cells. (A) The levels of H₂O₂ in the medium were determined by xylene orange assay and expressed as nmole/ml. The cells were cultured in the presence or absence of *H. pylori* (bacterium/cell ratio of 300:1) for 15, 30, and 60 min. (B) For the effects of 15d-PGJ₂, AG490, and DPI on *H. pylori*-induced H₂O₂ production, the cells were treated with 15d-PGJ₂ (10 μ M), AG490 (40 μ M), and DPI (10 μ M) for 2 hours, and cultured in the presence of *H. pylori* for 30 min. Values are means \pm S.E. * $P < 0.05$ compared with corresponding none (the cells culture in the absence of *H. pylori*), + $P < 0.05$ compared with *H. pylori* control (without treatment 15d-PGJ₂, DPI, and AG490).

RESULTS

H₂O₂ levels in the medium released from H. pylori-infected AGS cells

H₂O₂ levels in the medium released from *H. pylori*-infected AGS cells were determined during 60 min-culture period (Fig. 1A). Without *H. pylori*, the cells produced very low levels of H₂O₂ at each time point. With *H. pylori*, H₂O₂ levels in the medium increased from 15 min which continued up to 60 min. Therefore, to determine the effect of 15d-PGJ₂, DPI, and AG490 on *H. pylori*-induced H₂O₂ production, the cells were treated with 15d-PGJ₂, AG490, and DPI for 2 hours and cultured in the presence of *H. pylori* for 30 min (Fig. 1B). Both 15d-PGJ₂ and DPI, an NADPH oxidase inhibitor, inhibited *H. pylori*-induced increase in H₂O₂ levels, but AG490, a Jak/Stat3 inhibitor, has no effect on H₂O₂ production in *H. pylori*-infected AGS cells. H₂O₂ levels in the medium released from *H. pylori*-uninfected AGS cells (None) were not changed by treatment of 15d-PGJ₂, DPI, and AG490.

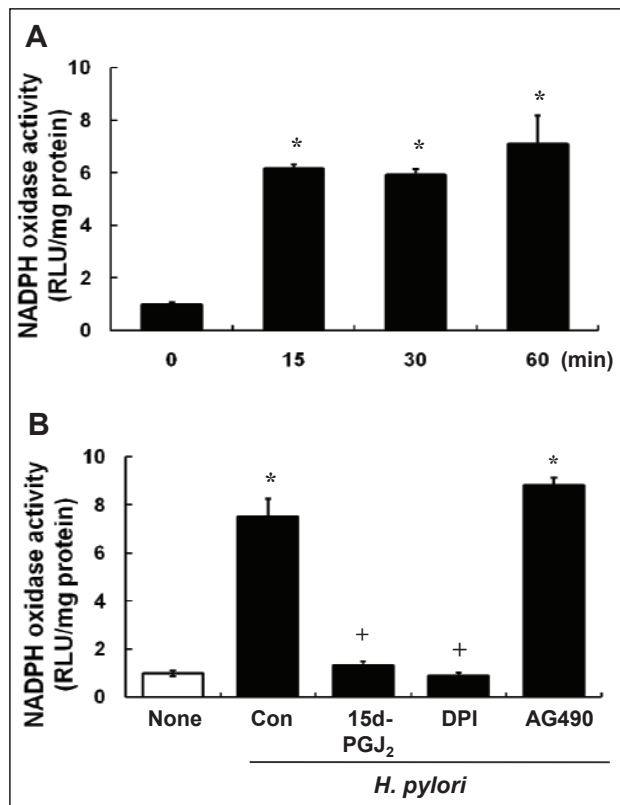


Fig. 2. NADPH oxidase activity of *H. pylori*-infected AGS cells. (A) The NADPH oxidase activities were determined by lucigenin assay. The cells were cultured in the presence or absence of *H. pylori* (bacterium/cell ratio of 300:1) for 15, 30, and 60 min. Membrane fractions of *H. pylori*-infected AGS cells were added to 10 μ M lucigenin to determine NADPH oxidase activity and cytosolic fractions were used for the negative control. NADPH oxidase activity was expressed as RLU/mg protein. (B) To determine the effect of 15d-PGJ₂, DPI, and AG490 on *H. pylori*-induced increase in NADPH oxidase activity, the cells were treated with 15d-PGJ₂ (10 μ M), AG490 (40 μ M), and DPI (10 μ M) for 2 hours and cultured in the presence of *H. pylori* for 15 min. Values are means \pm S.E. * P <0.05 compared with 0 min (A) or none (B, the cells culture in the absence of *H. pylori*). + P <0.05 compared with *H. pylori* control (without treatment of 15d-PGJ₂, DPI, and AG490).

NADPH oxidase activity of H. pylori-infected AGS cells

By monitoring ROS production with lucigenin luminescence from the membrane fractions of the cells infected with *H. pylori*, NADPH oxidase activity of *H. pylori*-infected cells was determined (Fig. 2). The cells have a relatively low activity of NADPH oxidase at the start of the experiment (0 min). *H. pylori* substantially stimulated NADPH oxidase activity of the cells from 15 min infection of *H. pylori*, which continued up to 60 min (Fig. 2A). To determine the effect of 15d-PGJ₂, DPI, and AG490 on *H. pylori*-induced increase in NADPH oxidase, the cells were treated with 15d-PGJ₂, AG490, and DPI for 2 hours and cultured in the presence of *H. pylori* for 15 min (Fig. 2B). *H. pylori*-induced increase in NADPH oxidase activity was significantly suppressed by 15d-PGJ₂. Similar effect was shown by DPI. However, AG490 had no effect on *H. pylori*-induced increase in NADPH oxidase activity, which was similar to its effect on *H. pylori*-induced increase in H₂O₂ levels of AGS cells (Fig. 1B). Therefore, NADPH oxidase may be an upstream signaling for Jak/Stat3 activation in *H. pylori*-infected AGS cells.

NADPH oxidase activation of Helicobacter pylori-infected AGS cells

For NADPH oxidase activation, the translocation of cytosolic subunits p47^{phox} and p67^{phox} to the membrane is required. As shown in Fig. 3A, p47^{phox} and p67^{phox} in cytosol were translocated to membrane at 15 min after *H. pylori* infection. After 15 min of infection with *H. pylori*, p67^{phox} and p47^{phox} in the membrane tended to return to the cytosol. Actin as a loading control was not changed by *H. pylori* (Fig. 3A). To determine the effect of 15d-PGJ₂, DPI, and AG490 on *H. pylori*-induced activation of NADPH oxidase, the cells were treated with 15d-PGJ₂, AG490, and DPI for 2 hours and cultured in the presence of *H. pylori* for 15 min (Fig. 3B). *H. pylori*-induced translocation of cytosolic subunits, p47^{phox} and p67^{phox}, to membrane was inhibited by 15d-PGJ₂ and DPI. However, AG490 had no effect on *H. pylori*-induced activation of NADPH in AGS cells.

Jak1/Stat3 activation and Jak3-DNA binding activity of Helicobacter pylori-infected AGS cells

The activation of Jak1/Stat3 of *H. pylori*-infected cells was determined by phosphorylation of Jak1 and Stat3 of the

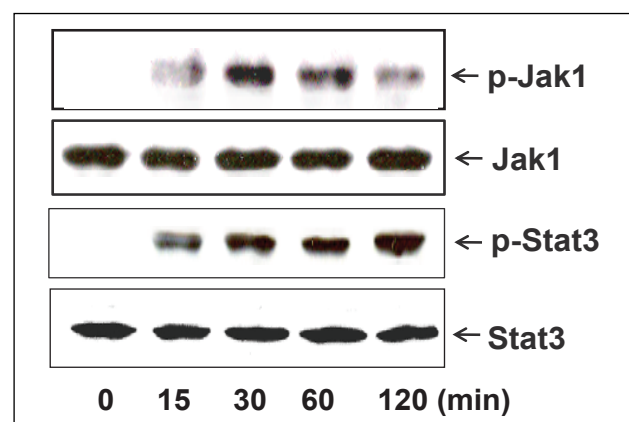


Fig. 4. Time-dependent activation of Jak1/Stat3 of *H. pylori*-infected AGS cells. The cells were cultured in the presence or absence of *H. pylori* (bacterium/cell ratio of 300:1) for 15, 30, 60, and 120 min. Whole cell extracts were prepared from *H. pylori*-infected cells. Phospho-specific and total forms of Jak1 and Stat3 were determined by Western blot analysis.

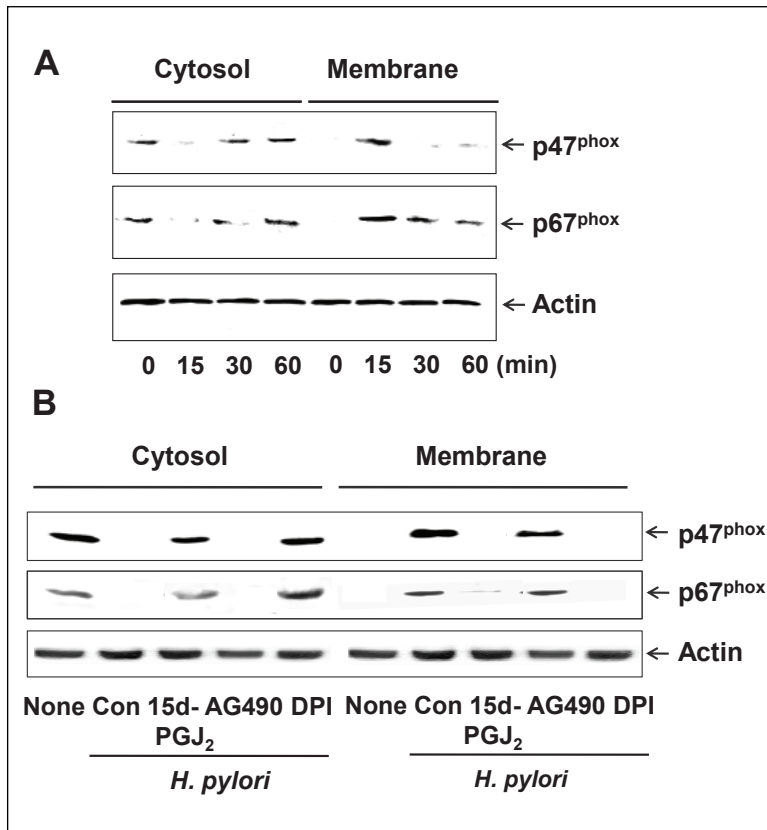


Fig. 3. NADPH oxidase activation of *H. pylori*-infected AGS cells. (A) The cells were cultured in the presence or absence of *H. pylori* (bacterium/cell ratio of 300:1) for 15, 30, and 60 min. Protein levels of p47^{phox} and p67^{phox} in cytosolic and membrane fractions were determined by Western blot analysis. Actin served as a loading control. (B) To determine the effect of 15d-PGJ₂, DPI, and AG490 on *H. pylori*-induced activation of NADPH oxidase, the cells were treated with 15d-PGJ₂ (10 μ M), AG490 (40 μ M), and DPI (10 μ M) for 2 hours and cultured in the presence of *H. pylori* for 15 min.

cells. Phospho-specific forms of Jak1 and Stat3 were observed at 15 min of *H. pylori* infection, which increased until 120 min (Fig. 4) while total forms of Jak1 and Stat3 were not changed by *H. pylori*. To determine the effect of 15d-PGJ₂, DPI, and AG490 on *H. pylori*-induced activation of Jak1/Stat3 and increase in Jak3-DNA binding activity of AGS cells, the cells were treated with 15d-PGJ₂, AG490, and DPI for 2 hours and cultured in the presence of *H. pylori* for 30 min (Fig. 5). 15d-PGJ₂, DPI, and AG490 inhibited *H. pylori*-induced phosphorylation of Jak/Stat3 (Fig. 5A) and Stat3-DNA binding activity (Fig. 5B) of AGS cells. Since DPI, an inhibitor of NADPH oxidase, suppressed the activation of Jak1/Stat3 similar to AG490, a Jak/Stat inhibitor, NADPH oxidase may regulate the activation of Jak1/Stat3 signaling in *H. pylori*-infected AGS cells. Taken together, 15d-PGJ₂ suppresses the activation of NADPH oxidase and the production of H₂O₂, which activates Jak1/Stat3 in *H. pylori*-infected gastric epithelial cells.

RANTES levels in the medium released from *Helicobacter pylori*-infected AGS cells

H. pylori increased the levels of RANTES in the medium time-dependently until 24 hours (Fig. 6A). To investigate the effect of 15d-PGJ₂, DPI, and AG490 on *H. pylori*-induced increase in RANTES in the medium released from AGS cells, the cells were treated with 15d-PGJ₂, AG490, and DPI for 2 hours and cultured in the presence of *H. pylori* for 4 hours (Fig. 6B). 15d-PGJ₂, AG490, and DPI significantly suppressed *H. pylori*-induced increase in RANTES in the medium released from AGS cells. The results show the involvement of NADPH oxidase and Jak1/Stat3 in RANTES production in *H. pylori*-infected AGS cells. Without *H. pylori*, 15d-PGJ₂, AG490, and DPI did not affect the production of RANTES in AGS cells.

DISCUSSION

H. pylori-induced inflammatory signalings have been focused on the activation of NF- κ B and AP-1 and IL-8 expression in gastric epithelial cells (9, 10, 32-34). In the present study, we found that Jak/Stat inflammatory signaling may mediate the expression of cytokines such as RANTES in *H. pylori*-infected cells. Even though Jak/Stat signaling has been widely studied on nervous system (40), the role of Jak/Stat activation in gastric diseases associated with *H. pylori* infection has not been well established. In the present study, we suggest that Jak/Stat activation may have a critical role in *H. pylori*-induced gastric inflammation by inducing cytokines including RANTES in gastric epithelial cells.

Previously, we demonstrated that *H. pylori*-induced production of ROS mediates the activation of NF- κ B and AP-1 and induction of IL-8 in gastric epithelial cells (10). In the present study, *H. pylori* induced increase in NADPH oxidase activity and translocation of NADPH oxidase cytosolic subunits, p67^{phox} and p47^{phox}, to membrane in gastric epithelial AGS cells. *H. pylori*-induced activation of NADPH oxidase and H₂O₂ production were inhibited by DPI, an inhibitor of NADPH oxidase. The results suggest that the source of ROS is NADPH oxidase in *H. pylori*-infected gastric epithelial cells. The present results were supported by our previous study showing that interaction of HSP90 with Rac1 activates NADPH oxidase in the membrane of *H. pylori*-infected gastric epithelial cells (15).

The activation of Jak1 and Stat3 plays an important role in the initiation of inflammation through the expression of inflammatory genes such as chemokines, cytokines, and adhesion molecules. All of these products initiate recruitment and activation of inflammatory cells and induction of inflammation. In the present study, AG490, an inhibitor of Jak/Stat3, did not inhibit *H. pylori*-induced H₂O₂ production,

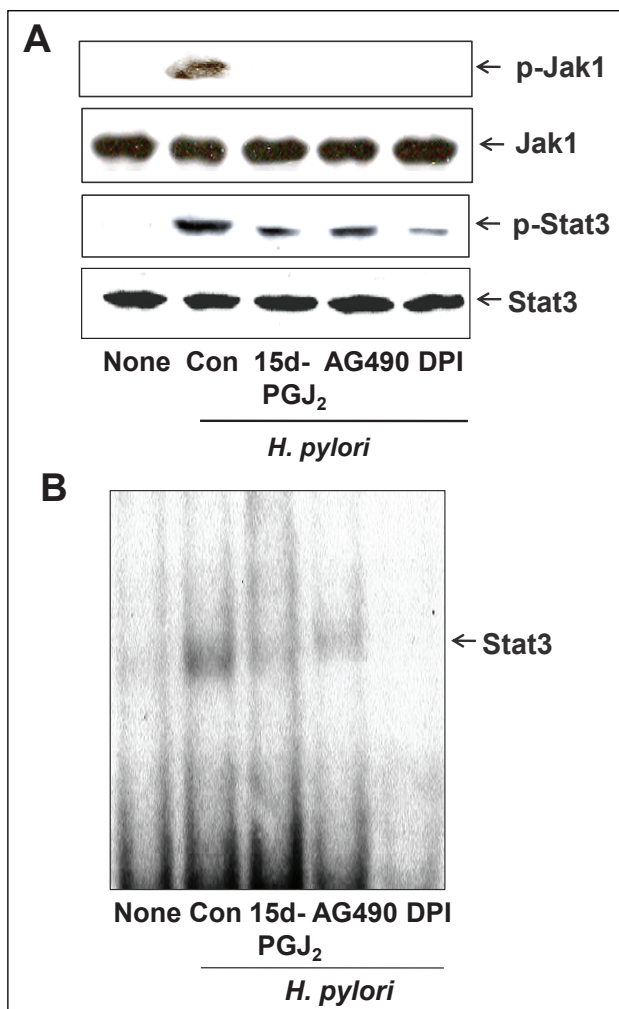


Fig. 5. The effects of 15d-PGJ₂, DPI, and AG490 on Jak1/Stat3 activation and Stat3-DNA binding activity of *H. pylori*-infected AGS cells. (A) The cells were treated with 15d-PGJ₂ (10 μ M), DPI (10 μ M), and AG490 (40 μ M) for 2 hours and cultured in the presence of *H. pylori* (bacterium/cell ratio of 300:1) for 30 min. Phospho-specific and total forms of Jak1 and Stat3 in whole cell extracts were determined by Western blot analysis. (B) DNA-binding activity of Stat3 in nuclear extracts was determined by EMSA.

NADPH oxidase activity, and translocation of p67^{phox} and p47^{phox} to the membrane, which was induced by *H. pylori*. The results demonstrate that Jak1 and Stat3 activation is the downstream signaling of NADPH oxidase which is activated by *H. pylori* in gastric epithelial cells.

Regarding the uptake of 15d-PGJ₂ into the cells, prostaglandins transported into the cells by a carrier-mediated active transport system (40-42). In the cells, 15d-PGJ₂ binds to PPAR- γ , but in some cases, it induces cellular responses devoid of PPAR- γ (43). Some of these effects have been reported to be mediated through covalent interaction of 15d-PGJ₂ with intracellular proteins such as I κ B kinase (44) and inhibit extracellular signal-regulated kinase (ERK) signaling pathway (45). Novel finding of the present study is that the 15d-PGJ₂ inhibits the activation of NADPH oxidase, which may be the starting point of *H. pylori*-induced inflammation.

In the studies of PPAR- γ ligands on gastric inflammation, the protective effect of PPAR- γ ligands, pioglitazone or 15d-PGJ₂, on gastric ischemia-reperfusion injury was reported in rats treated

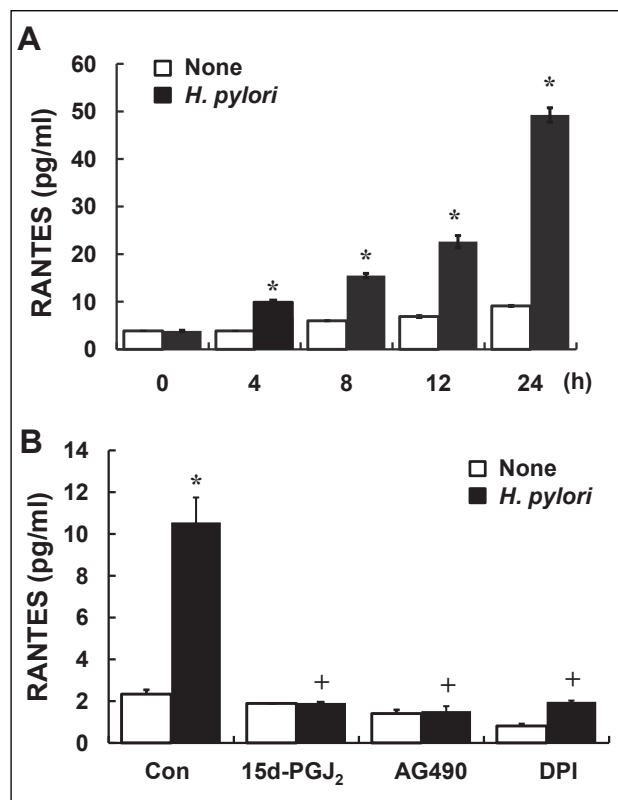


Fig. 6. RANTES levels in the medium released from *H. pylori*-infected AGS cells. (A) The level of RANTES in the medium was determined by ELISA and expressed as pg/ml. The cells were cultured in the presence or absence of *H. pylori* (bacterium/cell ratio of 300:1) for 4, 8, 12, and 24 hours. (B) To determine the effect of 15d-PGJ₂, DPI, and AG490 on *H. pylori*-induced production of RANTES, the cells were treated with 15d-PGJ₂ (10 μ M), AG490 (40 μ M), and DPI (10 μ M) for 2 hours and cultured in the presence of *H. pylori* for 4 hours. Values are means \pm S.E. * P <0.05 compared with the corresponding none (the cells culture in the absence of *H. pylori*). + P <0.05 compared with *H. pylori* control (without treatment of 15d-PGJ₂, DPI, and AG490).

ischemia-reperfusion *via* up-regulation of heat shock proteins and endoplasmic reticulum-related proteins (46) as well as inhibiting the induction of cyclooxygenase-2 (47). In addition, pioglitazone accelerates the healing of preexisting gastric ulcers of rats due to the anti-inflammatory action including suppression of interleukin-1 β , TNF- α , cyclooxygenase-2 and iNOS and overexpression of HSP70 (48). PPAR- γ ligands inhibit inflammatory gene transcription by inhibiting NF- κ B activation by PPAR- γ -dependent and independent mechanisms (49-51). A PPAR- γ antagonist GW9662 reversed protective effect of 15d-PGJ₂ on acute gastric mucosal injury, suggesting that 15d-PGJ₂ inhibits severity of gastric damage and expression of inflammatory cytokines such as TNF- α through PPAR- γ -dependent mechanisms (52). Slomiany have demonstrated that *H. pylori* lipopolysaccharide- (LPS-) elicited mucosal inflammatory responses were accompanied by a massive epithelial cell apoptosis, upregulation of iNOS, and COX-2 expression, and PPAR. PPAR- γ ligand ciglitazone suppresses these gastric mucosal inflammatory responses and may provide therapeutic benefits such as the amelioration of inflammation associated with *H. pylori* infection (53).

In the present study, we used a Jak/Stat3 specific inhibitor AG490 and an inhibitor of NADPH oxidase diphenyleneiodonium

(DPI) to determine the direct involvement of Jak/Stat and NADPH oxidase on the production of H_2O_2 and RANTES in *H. pylori*-infected cells. We found that DPI suppressed *H. pylori*-induced alterations similar to 15d-PGJ₂. However, AG490 had no effect on NADPH oxidase activation, but reduced the level of RANTES in the medium released from *H. pylori*-infected cells. The results show that NADPH oxidase activation is an upstream signaling of Jak1/Stat3 activation in *H. pylori*-infected AGS cells. ROS produced by NADPH oxidase activate inflammatory signaling including Jak1/Stat3 which induces the expression of RANTES in *H. pylori*-infected AGS cells. 15d-PGJ₂ inhibits the activations of NADPH oxidase and Jak1/Stat3 and RANTES expression, suggesting that 15d-PGJ₂ may be beneficial for the treatment of *H. pylori*-induced gastric inflammation. However, we could not conclude whether the inhibitory effect of 15d-PGJ₂ on the activations of NADPH oxidase and Jak1/Stat3 and induction of RANTES in *H. pylori*-infected gastric epithelial cells is through PPAR- γ activation or not. Further study should be performed using a PPAR- γ antagonist to determine the involvement of PPAR- γ in the effect of 15d-PGJ₂ on the protection of gastric epithelial cells from *H. pylori* infection.

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